MOLECULAR AND CELLULAR BIOLOGY, Apr. 1986, p. 1002–1009
0270-7306/86/041002-08$02.00/0

Vol. 6, No. 4

Monoclonal Antibody Y13-259 Recognizes an Epitope of the p21 *ras* Molecule Not Directly Involved in the GTP-Binding Activity of the Protein

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Received 12 November 1985/Accepted 3 January 1986

The p21 products of *ras* proto-oncogenes are GTP-binding proteins with associated GTPase activity. Recent studies have indicated that *ras* p21 may be required for the initiation of normal cell DNA synthesis, since microinjection of a monoclonal antibody, Y13-259, blocks serum stimulation of DNA synthesis in quiescent cell cultures (L. S. Mulcahy, M. R. Smith, and D. W. Stacey, Nature [London] 313:241–243, 1985). We localized the structural domain within the p21 molecule recognized by the Y13-259 monoclonal antibody. By analysis of a series of bacterially expressed p21 deletion mutants, the monoclonal antibody was found to interact with a region between positions 70 and 89 in the p21 amino acid sequence. By comparison of the coding sequences of different p21 proteins recognized by this monoclonal antibody, a highly conserved amino acid region between positions 70 and 81 was found to be the most likely site for the epitope detected by the Y13-259 antibody. This monoclonal antibody was further shown not to interfere directly with in vitro biochemical functions of the molecule, including GTP binding, GTPase, and autokinase activities.

A small set of eucaryotic genes, termed proto-oncogenes, can be activated as oncogenes by a variety of mechanisms in naturally occurring tumor cells (1, 5, 34). *ras* genes represent one of the most intensively studied families of proto-oncogenes. These genes were initially detected as the transforming genes of retroviruses (7). Recent evidence has implicated their activation as oncogenes in as many as 10 to 30% of human malignancies by mechanisms involving point mutations at one of two major sites in their coding sequence (2, 3, 13, 22, 24, 27, 32, 33, 35, 36). The structural and functional characterization of *ras* genes has been significantly aided by expression of their p21 products at high levels in *Escherichia coli* (14, 15, 30). Purification from bacterial extracts of normal and transforming p21s has been successfully achieved (14, 31). Moreover, microinjection of low concentrations of the activated p21 product, or of much higher concentrations of normal p21, has been shown to induce morphological alterations and DNA synthesis in quiescent cells (8, 29).

Biochemical activities associated with p21 proteins include GTP binding, GTP-dependent autophosphorylation, and GTPase activities (11, 16, 18, 25, 26). Recently, it has been shown that microinjection of a monoclonal antibody, designated Y13-259, which recognizes a shared epitope of *ras* p21 proteins specifically blocks the serum-induced mitogenic response of tissue culture cells (19). These findings imply that this antibody interacts with an important domain of the normal p21 molecule. We report here localization of the epitope recognized by the Y13-259 antibody to a small amino acid stretch that is shown not to be directly responsible for the nucleotide-binding, GTPase, or autokinase functions of the molecule.

MATERIALS AND METHODS

Bacterial strains. *E. coli* N4830 (P-L Biochemicals, Inc., Milwaukee, Wis.) was used for the expression of the *ras*-generated p21 products as described previously for *E. coli* RRI (14). The N4830 strain carries the temperature-sensitive *c*I857 gene and the *N* gene of lambda lysogen to give a thermoinducible expression system. Expression of *ras* proteins was obtained in NZY (14) broth at 42°C for 1 h, and bacteria cells were processed as indicated below.

Plasmid constructions. Digestion of DNAs was performed following the instructions of the supplier (New England BioLabs, Inc., Beverly, Mass.). Dephosphorylation of digested plasmids was carried out with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 10 mM Tris hydrochloride–10 mM $MgCl_2$ (pH 8.5) for 1 h. Ligation of inserts to plasmids or synthetic linkers to DNA fragments was performed in 60 mM Tris hydrochloride (pH 7.6)–10 mM $MgCl_2$–10 mM dithiothreitol–1 mM ATP–1 mM spermidine at 14°C for 14 to 16 h. Bacterial transformations were carried out as previously indicated (14).

Protein expression and analysis. Bacterial cells containing *ras* gene expression vectors were grown in NZY broth supplemented with 50 μg of ampicillin per ml at 30°C. When an $A_{590} = 0.2$ was reached, cells were transferred to 42°C and incubated at 250 rpm for 1 h. After centrifugation at 2,500 rpm for 10 min in a Sorvall RT6000 centrifuge at 4°C, pellets were washed twice in 30 mM Tris hydrochloride–5 mM EDTA–100 mM NaCl (pH 7.5) and sonicated for 30 s. After centrifugation at 12,000 rpm for 10 min in a Sorvall SS34 rotor, *ras* p21 proteins were solubilized in 7 M urea–20 mM Tris hydrochloride (pH 7.5) and centrifuged at 30,000 rpm for 30 min in a Beckman ultracentrifuge. Supernatants were collected and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in equal volumes of sample buffer as indicated in the legend to Fig. 3. When indicated, bacterial cells were grown in M9 (14) broth supplemented with a mixture of amino acids as indicated above. When transferred to 42°C for the induction of *ras* protein expression, the cells were transferred to M9 medium without methionine. After 5 min at 42°C, [^{35}S]methionine (800 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to a final concentration of 50 μCi/ml. Cells were

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TABLE 1. GTP-binding activity of *E. coli*-expressed *ras* p21s after immunoprecipitation with different antibodies[a]

Sample	Origin	cpm with the following serum:				
		NRS	Y13-259	YA6-172	Polyclonal	M3
p21-H (Lys-12–Ala-59)	BALB-MSV	1,354	205,467	204,465	2,032	NT[b]
p21-H (Arg-12–Thr-59)	Harvey-MSV	2,033	818,017	819,204	3,528	3,795
p21-K (Ser-12–Thr-59)	Kirsten-MSV	1,294	500,418	2,186	6,577	NT

[a] Approximately 2 μg (2.5 μl) of *E. coli* extracts containing p21 proteins was immunoprecipitated as described previously with an excess of normal rabbit serum, rabbit polyclonal anti-p21, and monoclonal antibody YA6-172 or Y13-259 in 400 μl (total volume) of Staph A buffer (28). The amounts of antisera utilized were first standardized by their ability to remove all active p21 molecules from supernatants (Fig. 1; data not shown). After washing three times with GTP binding buffer, immunoprecipitates were resuspended in 200 μl of the same buffer containing 1.67 pmol of [α-^{32}P]GTP (~3,000 Ci/mmol; ICN Pharmaceuticals) per assay. Incubation was performed for 30 min at 37°C, and pellets were washed three times with cold GTP binding buffer. After the last wash, pellets were resuspended in 200 μl of same buffer, 10 ml of Aquasol was added, and samples were counted in a scintillation counter. Total counts per minute are shown, with 1.82×10^6 cpm equivalent to 1 pmol of [α-^{32}P]GTP.

[b] NT, Not tested.

incubated for 1 h and then collected and processed as indicated above.

GTP-binding activity. GTP binding of p21 *ras* proteins was measured by incubation (30 min at 37°C) of immunoprecipitates, obtained as described in footnote *a* of Table 1, in 50 mM Tris hydrochloride (pH 7.5)–10 mM $MgCl_2$–2.5 mM dithiothreitol–100 μg of bovine serum albumin per ml plus ~2 pmol of [α-^{32}P]GTP per assay. After washing three times, the pellets were resuspended in 200 μl of the same buffer, and radioactivity retained in the immunoprecipitates was determined by scintillation counting. GTP-binding activity of highly purified p21 proteins was assayed as well by the amount of radioactivity retained in nitrocellulose filters (BA85; Schleicher & Schuell, Inc., Keene, N.H.) after incubating different amounts of p21 proteins under the same conditions as above. Samples were filtered and washed twice with 10 ml of cold GTP binding buffer as before, and the radioactivity retained by the filter was counted in a scintillation counter.

Autophosphorylation of *ras* p21 proteins. Approximately 25 ng of highly purified *ras* p21 proteins was immunoprecipitated as described below and then incubated at 37°C for 1 h in GTP binding buffer containing ~3.34 pmol of [γ-^{32}P]GTP (3,048 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.). After washing three times with the same buffer, proteins were resuspended in sample buffer for 5 min at 90°C and applied to a 12.5% SDS-polyacrylamide gel as indicated in the legend to Fig. 2.

Immunoprecipitation of p21 *ras* proteins. After expression of the various p21 *ras* proteins as described above, samples of solubilized proteins were diluted 50 times in 200 μl of 10 mM sodium phosphate–1% Triton X-100–0.1% SDS–0.5% sodium phosphate–1 mM phenylmethyl-sulfonyl fluoride–aprotinin (100 Kalikrein inactivator units per ml), pH 7.4. Extracts were incubated for 30 min at 4°C with different amounts of anti-Harvey murine sarcoma virus (MSV) p21 monoclonal antibodies, polyclonal antibody, or preimmune rat serum. Protein A-Sepharose was swollen and washed in

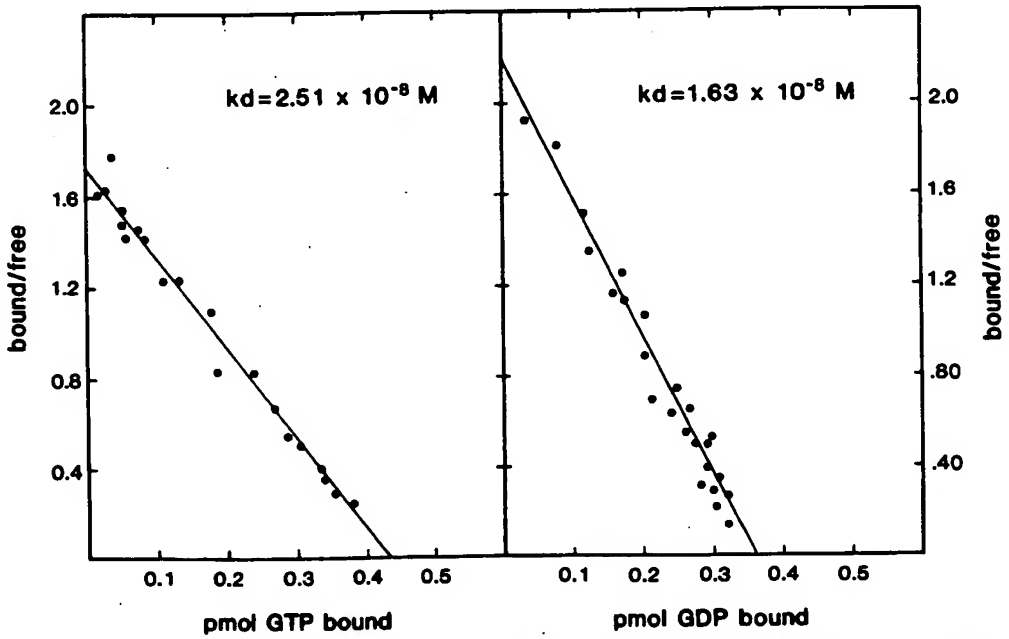

FIG. 1. Scatchard analysis of guanine nucleotide binding to the purified *ras* p21 protein of Harvey-MSV. H-*ras* p21 expressed in *E. coli* was incubated with various concentrations (10^{-9} to 10^{-6} M) of either [^{3}H]GDP (11,500 cpm/pmol) (right) or [α-^{32}P]GTP (3.2×10^5 cpm/pmol) (left) in 200 μl of binding buffer as indicated in Materials and Methods. Incubation was performed for 2 h at 0°C and then for 30 min at 37°C. The amount of nucleotide bound to the protein, as determined by retention on nitrocellulose filters, was measured by scintillation counting.

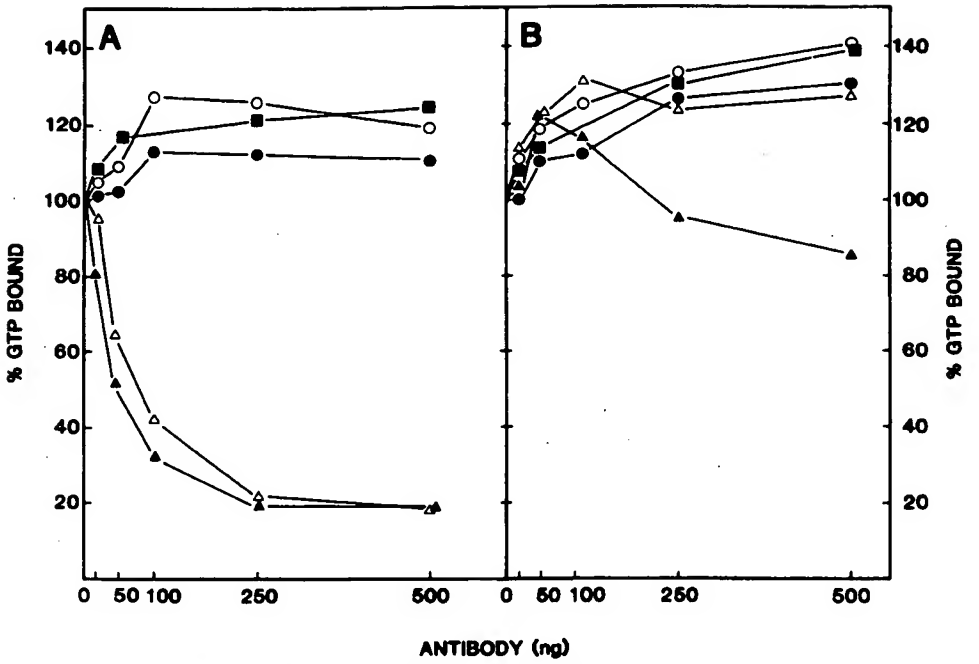

FIG. 2. (A) A 25-ng sample of purified bacterially expressed Harvey *ras* p21 was incubated on ice for 30 min with different amounts of ammonium sulfate-purified antisera in 390 μl of GTP binding buffer. A 10-μl portion of the same buffer containing 1.67 pmol of [α-^{32}P]GTP was then added, and incubation continued at 37°C for 20 min. The reaction was stopped by the addition of 10 ml of ice-chilled GTP binding buffer to each sample. Samples were filtered through nitrocellulose filters (BA85) and washed twice with 10 ml of cold GTP binding buffer, and the radioactivity retained by the filter was counted in a scintillation counter. The amount of [α-^{32}P]GTP-p21 bound to the filter in the absence of antiserum was standardized to 100% binding (mean of four reactions, 187,669 ± 13,500 cpm; 1 pmol ≃ 1.28 × 10^6 cpm). (B) A 25-ng sample of p21 protein as above was incubated in 200 μl of GTP binding buffer at 37°C for 45 min in the presence of 1.67 pmol of [α-^{32}P]GTP. After the addition of various amounts of antibody in 200 μl of the same buffer, the samples were incubated on ice for 30 min, processed, and filtered through BA85 nitrocellulose filters as indicated. The amount of [α-^{32}P]GTP-p21 bound to the filter was standardized to 100% as above (mean of four reactions, 181,075 ± 10,320 cpm; 1 pmol ≃ 1.28 × 10^6 cpm). A maximum 5 M ratio of antibody to p21 antigen was achieved for each antibody analyzed. Symbols: ●, NRS; ○, Y13-259; △, polyclonal antibody; ▲, M3 monoclonal antibody; ■, YA6-172.

the same buffer and coated with goat anti-rat or anti-mouse immunoglobulin G as described by Furth et al. (9). A 1:10 (wt/vol) suspension of the coated protein A-Sepharose (200 μl) was added to each sample, and the samples were shaken in an Eppendorf shaker at 4°C for 15 min. Immunocomplexes were washed three times with the same buffer, and the final pellets were dissolved at 90°C for 2 to 3 min in sample buffer. Samples were analyzed by SDS-polyacrylamide gel electrophoresis as described above.

TABLE 2. Effect of prebound GTP on immunoprecipitation of p21 by different antibodies[a]

Serum	cpm
NRS	645
Y13-259	196,780
YA6-172	217,725
Polyclonal	10,745
M3	3,705

[a] Samples (1 μg) of *E. coli* extracts containing p21 from Harvey-MSV were incubated in 200 μl of GTP binding buffer at 37°C for 60 min in the presence of 10 μM [α-^{32}P]GTP. Saturating amounts of each antibody were then added as described in Table 1, footnote *a*, and the incubation continued for 30 min on ice. Samples (200 μl) of a preparation of goat anti-rat immunoglobulin G-precoated protein-A sepharose (50 mg/ml) in the same buffer containing 1% Triton X-100 were added, and incubation continued at 4°C for 15 min in the presence of continuous shaking. Immunoprecipitates were washed three times with 1 ml of GTP binding buffer, and radioactivity retained in the immunoprecipitates was determined by scintillation counting. Total counts per minute are shown, with 9.5 × 10^5 cpm equivalent to 1 pmol of [α-^{32}P]GTP.

RESULTS

Analysis of in vitro activities of p21 *ras* proteins in the presence of monoclonal antibodies. To determine whether the p21 epitope recognized by the Y13-259 antibody was responsible for any of the known biochemical functions of the molecule, we compared the effects of Y13-259 with that of YA6-172 monoclonal antibody and polyclonal antibody prepared against purified p21 on these activities. Harvey, BALB, and Kirsten *ras* proteins, expressed as nonfused products in *E. coli*, were utilized for comparative analysis. Each of these proteins, purified as described below, was shown to exhibit transforming activity upon microinjection of ~2 × 10^5 to 3 × 10^5 molecules per cell into NIH/3T3 cells (data not shown).

The p21 products of BALB and Harvey *ras* genes demonstrated almost identical levels of binding of [α-^{32}P]GTP after immunoprecipitation with an excess of either Y13-259 or YA6-172 monoclonal antibody (Table 1). YA6-172 is known not to bind viral Kirsten *ras* p21 (9), resulting in the lack of detectable GTP binding of the Kirsten *ras* p21 in this experiment (bottom row). Under the same conditions of antibody excess, polyclonal antibody directed against bacterially expressed H-*ras* p21 completely inhibited GTP binding by all three p21 proteins. These results strongly suggested that a domain(s) of the p21 molecule required for

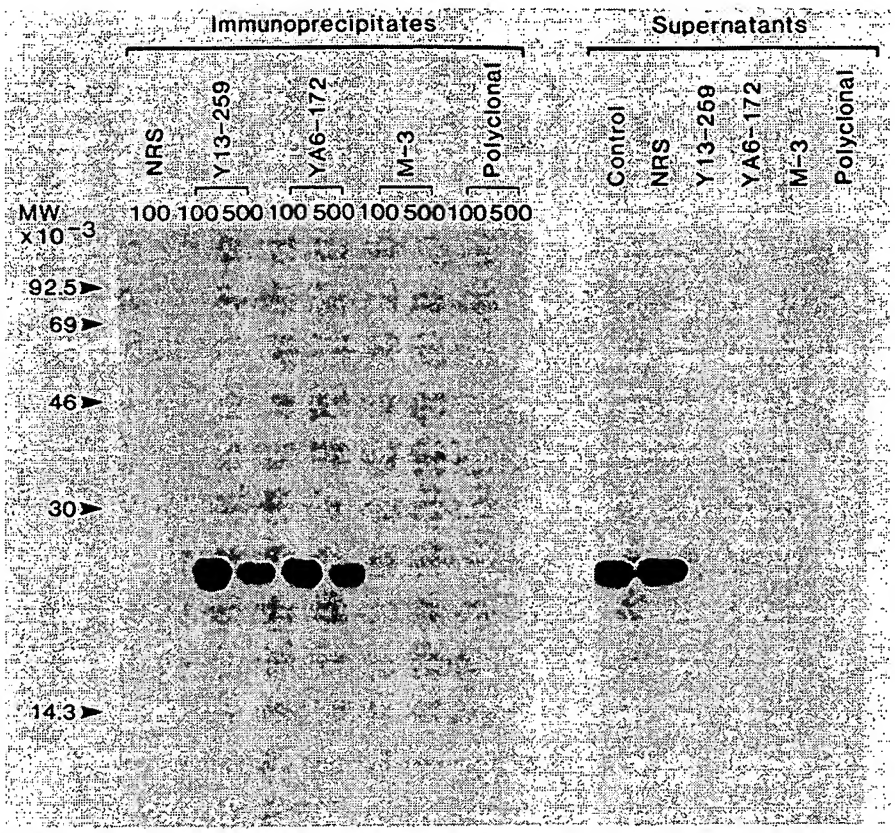

FIG. 3. A 25-ng sample of purified Harvey *ras* p21 was incubated on ice for 30 min with 100 or 500 ng of each ammonium sulfate-purified antiserum in 200 μl of GTP binding buffer. A 200-μl portion of a 50-mg/ml protein A-sepharose solution was then added, and incubation was allowed to proceed for 15 min at 4°C with continuous shaking. After centrifugation for 10 min at 12,000 rpm in a Beckman Microfuge, pellets and supernatants were separately analyzed for autophosphorylation at 37°C in the presence of ~3.34 pmol of [γ-^{32}P]GTP (3,048 Ci/mmol) for 1 h. Samples were then applied to a 12.5% polyacrylamide gel and processed for autoradiography as described elsewhere (28). NRS, Normal rat serum; C, without antiserum addition. Left panel, Autophosphorylated p21 after immunoprecipitation with 100 or 500 ng of antiserum. Right panel, Supernatants of samples treated with 100 ng of antiserum. MW, Molecular weight.

GTP binding can be blocked by antibody but that neither Y13-259 nor YA6-172 antibody recognized such a domain(s).

We utilized a different assay method as another approach to exclude the possibility of a direct interaction of the Y13-259 antibody with the GTP-binding domain of p21. By this approach, GTP bound to p21 in solution is measured by nitrocellulose filter binding (12). Bacterially expressed H-*ras* p21 (Arg-12–Thr-59) was solubilized from lysed bacteria utilizing 7 M urea and purified by means of Sephadex G-100 chromatography, as described in Materials and Methods. Fractions shown to be more than 95% pure in p21 were pooled and analyzed for known p21 activities. Scatchard plot analysis demonstrated stoichiometric binding for both GTP and GDP, consistent with single-site binding kinetics in both cases, with K_d values of 2.51×10^{-8} and 1.63×10^{-8} M, respectively (Fig. 1), in agreement with previously reported values (12).

Preincubation of the purified H-*ras* p21 protein with up to a 2.8 M ratio of either Y13-259 or YA6-172 antibody did not inhibit the GTP-binding properties of the protein (Fig. 2). This concentration is actually a 5.6-fold excess owing to the fact that the antibody is a bifunctional molecule. Both antibodies Y13-259 and YA6-172, as well as normal serum, induced a small but reproducible apparent increase in the amount of GTP bound. In contrast, preincubation with increasing amounts of the polyclonal antiserum or with M3, a mouse immunoglobulin G monoclonal antibody directed against H-*ras* p21 expressed in *E. coli* (J. C. Lacal and S. A. Aaronson, manuscript in preparation), was associated with progressive and striking inhibition of GTP binding (Fig. 2A). As a control, preincubation of p21 with labeled GTP before antibody addition was associated with no detectable inhibition by Y13-259 of the GTP-p21 complex detected by the filter assay (Fig. 2B).

From the above results, it might be reasoned that GTP bound to p21 would prevent recognition of the p21 molecule by an antibody which interacted directly with the GTP-binding site. When p21 was preincubated with [α-^{32}P]GTP at saturating concentration and subsequently immunoprecipitated with YA6-172 or Y13-259, there was no appreciable difference in the amount of GTP-p21 complex recognized (Table 2). In striking contrast, the monoclonal antibody M3, as well as the polyclonal antibody, both of which inhibited GTP binding by p21 (Fig. 2), failed to immunoprecipitate the GTP-p21 complex under the same conditions at which they were readily capable of immunoprecipitating p21 molecules to which GTP was not bound (see below). Since the polyclonal antibody failed to precipitate the GTP-p21 complex, the antigenic determinants recognized by this antiserum must predominantly be those involved in GTP binding. All of

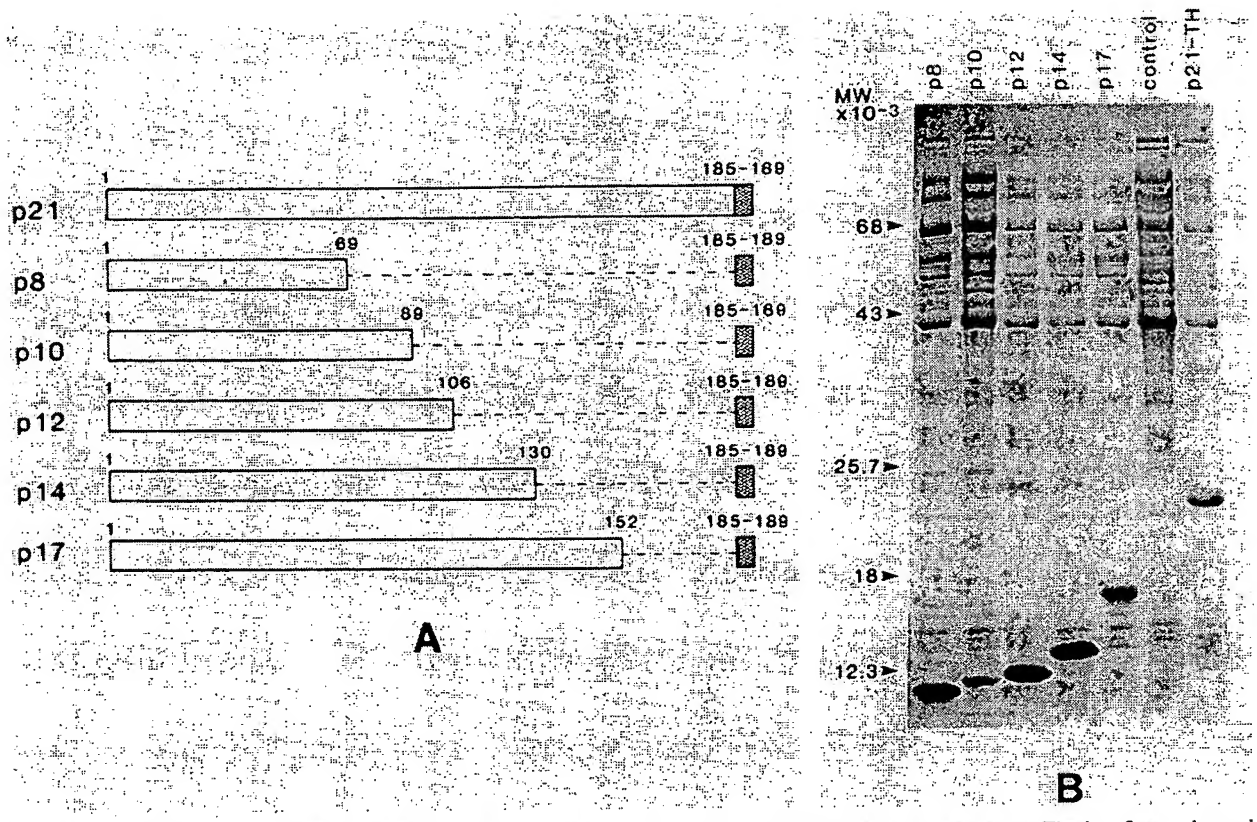

FIG. 4. (A) Diagram of the different proteins generated by deletions of variable regions of the coding sequence. The last five amino acids are present in all cases and are indicated as a striped box (▨). Numbers refer to the last amino acid before the deletion. (B) Bacterial clones expressing *ras* p21 or p21 deletion mutants in plasmid pJCL-41 (Lacal et al., in preparation) were grown up to A_{590} = 0.2 at 30°C in NZYDT medium plus 50 μg of ampicillin per ml. At that point cells were transferred to 42°C, and incubation proceeded for 60 min. Cells were collected and processed as described under Material and Methods. Solubilized samples were mixed to equal volumes of sample buffer and loaded onto 12.5% SDS-polyacrylamide gels. After electrophoresis, the gel was stained with Coomassie brilliant blue. The protein molecular weight (MW) markers were: bovine serum albumin (68,000); ovalbumin (46,000); α-chrymotrypsinogen (25,700); β-lactoglobulin (18,400); cytochrome *c* (12,300).

the above findings demonstrate that Y13-259 must recognize a domain of the p21 molecule distinct from its GTP-binding domain.

We next analyzed autokinase activity associated with the Harvey *ras* p21 protein. Under conditions in which a limiting amount of p21 was immunoprecipitated with different antisera, similar high levels of autokinase activity were observed with Y13-259 and YA6-172 antibodies (Fig. 3). In contrast, when p21 was first immunoprecipitated with either the M3 monoclonal antibody or the polyclonal antiserum, there was little or no detectable p21 autokinase activity. All of the antisera were shown (Fig. 2) to be in marked excess of the amount of p21 (25 ng) utilized in these experiments. This was confirmed by the absence of residual p21 autokinase activity in supernatants after p21 immunoprecipitation with any of the antisera (Fig. 3). Sweet et al. (31) have shown that p21 H-*ras* protein immunoprecipitated by the Y13-259 antibody possesses p21-associated GTPase activity. We confirmed these results as well with BALB- and Harvey-MSV p21 proteins immunoprecipitated by either the YA6-172 or the Y13-259 antibody. Both normal and transforming p21s, after immunoprecipitation with YA6-172, showed GTPase activity identical to that of p21s immunoprecipitated with Y13-259 antibody (data not shown). Thus, the three known biochemical activities of p21 molecules are associated with domains distinct from that recognized by the Y13-259 monoclonal antibody.

Localization of the epitope recognized by monoclonal antibody Y13-259 within the p21 molecule. Previous studies have indicated that Y13-259 recognizes a site distal to position 33 in the 189-amino acid chain (20). To precisely localize the epitope, we utilized a strategy based on the generation of a series of p21 deletion mutants expressed in bacteria. Each mutant protein contained the nonfused original amino terminus and the carboxy-terminal amino acids of the eucaryotic p21 molecule (Fig. 4), as will be described in detail elsewhere (Lacal et al., in preparation). The newly generated plasmids encoded p21 derivatives of approximately 8 (p8), 10 (p10), 12 (p12), 14 (p14), and 17 (p17) kilodaltons (Fig. 4).

Purified [^{35}S]methionine-labeled extracts from bacterial clones expressing p8, p10, p12, p14, p17, and p21 Harvey-*ras* proteins were immunoprecipitated with normal rabbit serum (NRS), rabbit anti-p21 polyclonal antibody, or Y13-259 monoclonal antibody. The parental p21 and each of the deletion mutants were detected by the polyclonal antibody (Fig. 5). Only p8 among the deletion mutants analyzed was not recognized by monoclonal antibody Y13-259. In addition, this antibody efficiently recognized deletion mutants of the p21 protein lacking residues 6 to 23, as well as a fused p21 product (14) in which the first four amino acids are

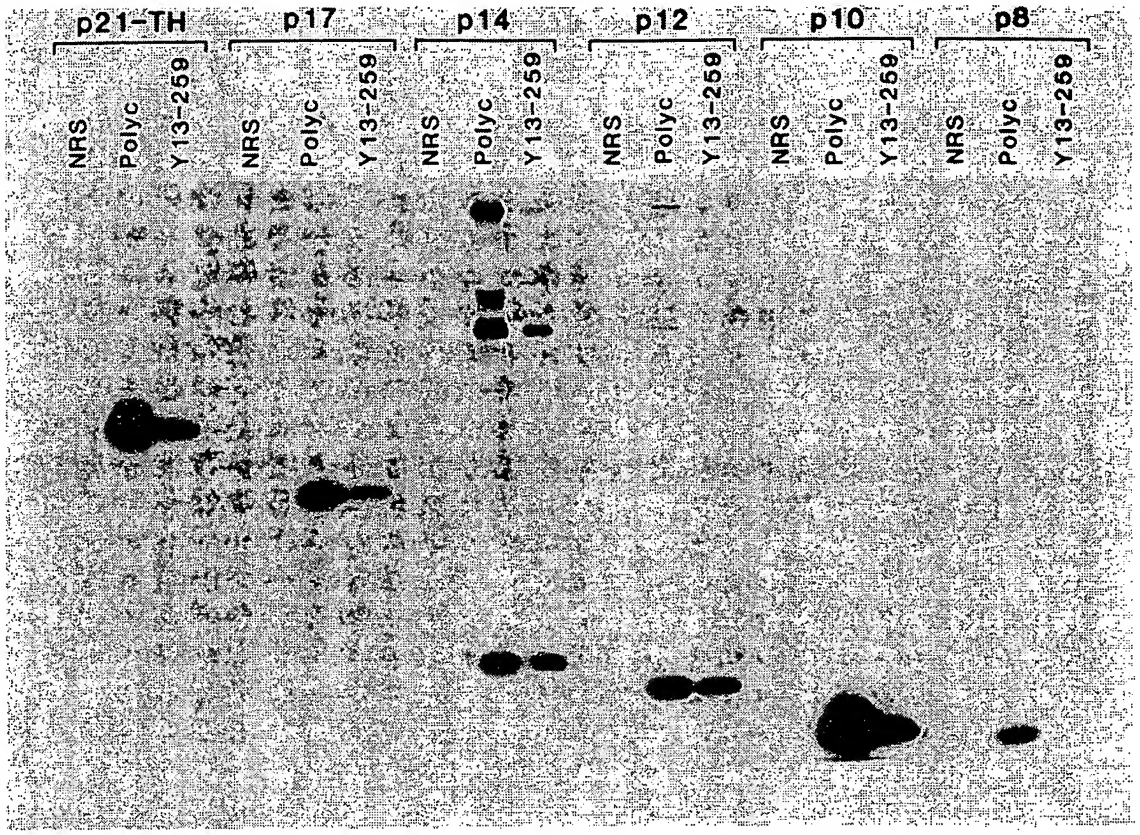

FIG. 5. Bacterial clones expressing p8, p10, p12, p14, p17, and p21-TH proteins were grown at 30°C as indicated in the legend to Fig. 3 in M9 medium with methionine. When transferred to 42°C, cells were changed to M9 medium without methionine, and [^{35}S]methionine (800 Ci/mmol) was added to a final concentration of 50 μCi/ml. After heat shock induction, cells were collected and processed as described in the legend to Fig. 3. Partially purified samples obtained as described in Table 1 were immunoprecipitated as before with 0.5 μg of NRS, polyclonal antibody (polyc), or Y13-259 monoclonal antibody. SDS (12.5%)-polyacrylamide gel electrophoresis and autoradiography were performed as described previously (28). Autoradiogram was obtained after 3 h of exposure at −70°C.

substituted by eight amino acids encoded by the expression plasmid (data not shown). The patterns of recognition of the truncated p21 proteins by YA6-172 and M3 monoclonal antibodies were distinct from that observed with Y13-259 (data not shown), implying that these monoclonal antibodies recognized different epitopes on the protein. The Y13-259 antibody has been shown to detect p21 molecules blotted to nitrocellulose paper after exposure to denaturing conditions

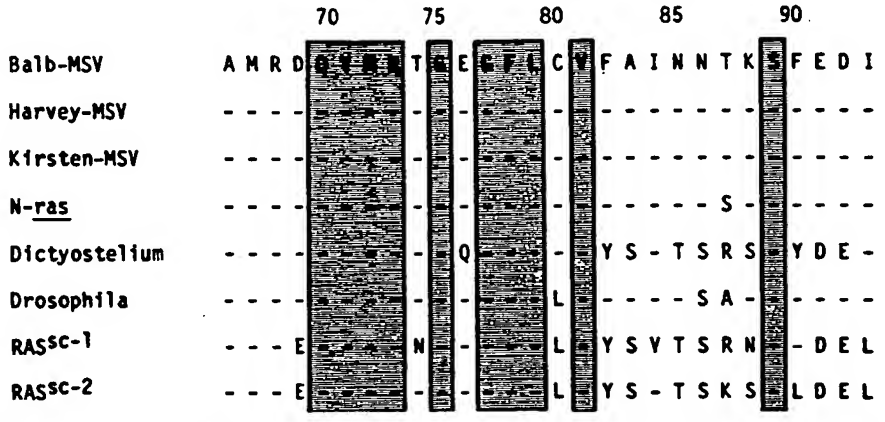

FIG. 6. Protein sequences of *ras* p21 proteins of different origins are compared in the region between amino acids 66 and 93. Boxes indicate regions of complete homology between residues 69 and 89 among all the known members of the *ras* family recognized by Y13-259 monoclonal antibody.

(14). All of these findings strongly suggest that Y13-259 recognizes the domain between positions 70 and 89 in the p21 coding sequence. A detailed comparison among different p21 *ras* family members shows that they exhibit some variability in their position 82 to 88 codons (Fig. 6). Since Y13-259 recognizes diverse p21s from yeasts to human (9, 20, 21, 28), the highly conserved region between residues 70 and 81 is the most likely site for Y13-259 antibody recognition.

DISCUSSION

A recent report has shown that microinjection of Y13-259 antibody specifically blocks serum-stimulated DNA synthesis in quiescent 3T3 cells (19). These findings imply that the site at which this antibody interacts with normal p21 must exert a profound inhibitory effect on a p21 function required for serum activation of normal cellular DNA synthesis. The nature of this interference is not yet understood, but findings that other p21 monoclonal antibodies lack this inhibitory activity have argued that Y13-259 has the specific ability to interact at a functionally important site within the molecule (19). We localized the site of p21 binding by this monoclonal antibody to a small region of around 10 amino acids at positions 70 to 81 in the p21 coding sequence. This represents a region of the molecule very highly conserved in *ras* proteins from yeasts to humans (6, 10, 21, 23) and in relatively close proximity to one of the two major sites at which point mutations are known to activate the oncogenic potential of *ras* proto-oncogenes (35).

Our present studies established that the Y13-259 antibody does not interact directly with the GTP-binding site of the p21 molecule or interfere with its associated GTPase or autokinase activities. Our evidence includes the inability of this antibody to inhibit GTP binding under different assay conditions in which other antibodies strikingly and reproducibly inhibited this biochemical function. Moreover, the p21-GTP complex was readily recognized by Y13-259, while other antibodies which interacted directly with the GTP-binding site failed to detect this complex.

While the results of some investigations have been consistent with our conclusions concerning the lack of effect of Y13-259 on GTP binding by p21 (4), there have been contradictory reports as well. Furth et al. (9) found that Y13-259 inhibited GTP binding in studies utilizing crude cell extracts, results that were not reproducible with more purified eucaryotic p21 preparations and highly purified antibody (Furth, personal communication). More recently, Hattori et al. (12) reported that Y13-259 inhibited GTP binding by their bacterially expressed, fused p21 protein. Scatchard analysis of our p21 protein and that of Hattori et al. (12) indicated comparable dissociation constants with single GTP-binding species for each. Thus, the explanation for the striking differences between the results of Hattori et al. and our own studies demonstrating lack of inhibition by Y13-259 of GTP binding by p21 is unlikely to reflect differences in affinities of the two proteins for GTP or the existence of different GTP-binding species. Whether the explanation relates to primary structure or conformational differences between their fused as compared with our nonfused p21 protein is not known. In this regard, it will be of interest to determine whether the p21 protein utilized by Hattori et al. is biologically active in vivo as has been demonstrated for our unfused p21 proteins (J. C. Lacal et al., Cell, in press).

McCormick et al. (17) have recently reported a model for the tertiary structure of p21 based on the known tertiary structure of the GDP-binding domain of the related elongation factor EF-Tu, a protein whose primary sequence shows some similarities to p21. According to this model, the GTP-binding region is made up of amino acid sequences from positions 10 to 16, 57 to 63, and 116 to 119, and probably sequences around amino acid 145. Our results are consistent with this model if the antibody Y13-259 recognizes sequences around 70 to 75, which in the model are exposed to the opposite side of the molecule to which the binding of GTP is proposed.

Since our findings demonstrate that Y13-259 does not directly interfere with the known in vitro biochemical functions of p21, including GTP-binding, GTPase, and autokinase activities, the antibody may affect some as yet undetected function such as substrate or modulator binding or other enzymatic activity. It will be of interest to directly assess the effects of site-directed lesions of this domain on the in vitro and in vivo functions of the p21 protein. The strategy used to identify this domain by using a series of bacterially expressed p21 deletion mutants may also be useful in localizing other important p21 functional domains recognized by monoclonal antibodies.

ACKNOWLEDGMENTS

We thank S. Srivastava for stimulating discussions and collaboration, Steven Anderson for technical assistance, and Holly Fuhrer for photographic work.

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